



Imperacer[®] Kit

for real-time PCR detection

Biotin Immuno-PCR Assay

Catalog Number 12-007 kit-R

For the quantitative determination of biotinylated antibodies or antigens.
Adaptable to various matrices.

This package insert must be read in its entirety before using this product.

**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.
DEVELOPMENT KIT – NOT FULLY OPTIMIZED**

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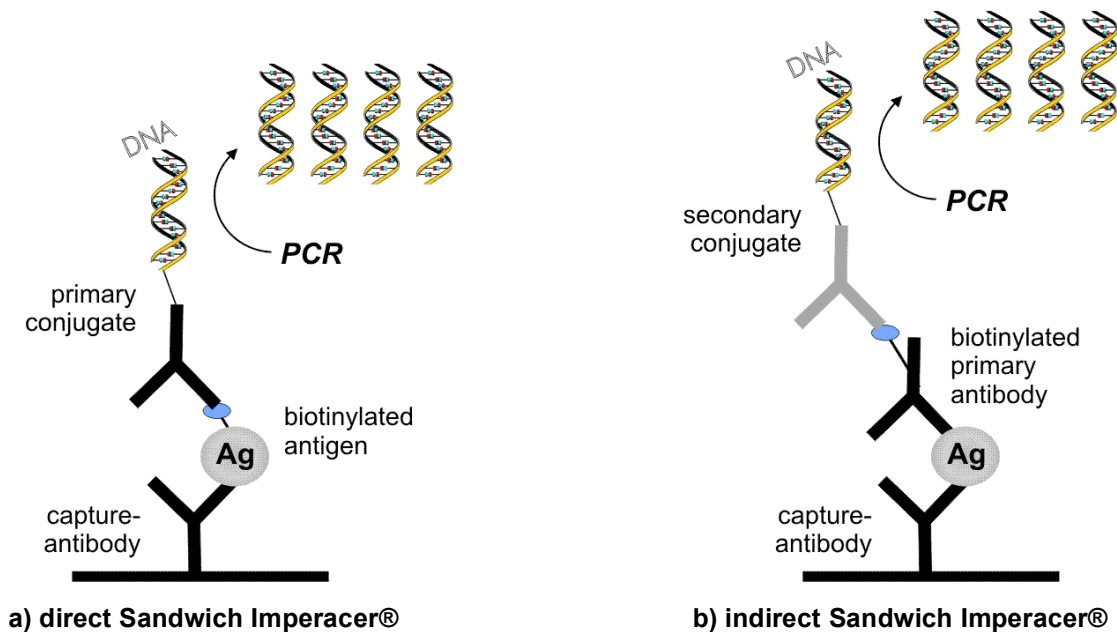
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PRINCIPLE OF THE ASSAY

Due to the high sensitivity of this kit, please note the following precautions.

- Use separate pipettes and glassware for dispensing all reagents to avoid cross-contamination.
- Careful washing of the microplate is essential to minimize nonspecific binding of the conjugate.



This assay employs the quantitative sandwich Immuno-PCR technique. The anti-biotin Imperacer® conjugate “*CHI-Biotin*” could be used as primary conjugate for the direct detection of biotinylated compounds in a sandwich assay (a) or as an indirect secondary conjugate against a biotinylated antigen-specific primary antibody (b). Signal read-out is done by real-time PCR.

Imperacer®: The model assay protocol in this manual gives the necessary instructions for both methods, a) and b). It could be adapted to a number of different applications by adapting the protocol and/or changing of reagents. We recommend to carry out an optimization round for each novel application, varying the key concentrations of the capture or primary antibodies and the Imperacer® conjugate, starting with the concentrations given in this protocol. It is also possible to stay as near to the standard ELISA protocol used previously with the reagent concentrations / incubation times used in ELISA and varying the conditions thereof.

It is recommended to make an optimization step for each novel matrix. Here, different dilutions of the matrix should be tested for optimal performance. For optimization a variation of the capture antibody and the Imperacer® conjugate concentration is recommended.

Amplification: Real-time PCR is carried out using a dual-labeled probe. The probe contains a fluorescence dye (Fam) and a quencher. While the DNA-polymerase

activity of the enzyme elongates the PCR-primer during the synthesis of the novel complementary DNA strand, the exonuclease activity of the enzyme reduces the probe bound next to the elongated primer. The separating of fluorescence marker and quencher thereby induces fluorescence for each amplified DNA strand. Signal readout of a real-time IPCR experiment is done according to the cyclor manual.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other sources or lots.
- Any variation in standard diluent, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Using different matrices can influence the kit performance regarding to the sample and collection and storage techniques.

REAGENTS

Material for one microplate, 1 x 96 wells

Microplate Modules	13 x (8 wells each)
Microplate Frame	2 x (reusable)
Adhesive Foil	2 x (for real-time PCR)
Buffer Reservoir	2 x
Coating Buffer	1 x 10 ml
Wash-Buffer A concentration *	1 x 50 ml
Wash-Buffer B concentration *	1 x 50 ml
Chimera Direct Block	1 x 25 ml
<u>AnySource® Sample Dilution Buffer</u>	
SDB ₁₀₀₀ Basic	4 x 1.5 ml

Ready-to-use aliquots for 16 wells (2 microplate modules) each:

Conjugate Dilution Buffer biotin free (CDB-b)	7 x 1.5 ml
Conjugate Dilution Buffer (CDB)	7 x 1.5 ml
PCR-Mastermix "I/Fam"	6 x 600 µl
Imperacer® conjugate "CHI-Biotin"	6 x 10 µl

*for reagent preparation see next page

STORAGE

	Unused Kit	Opened / Reconstituted Reagents
Coating Buffer	Store for up to 6 months at 2 – 8°C. *	Store for up to 1 month at 2 – 8°C. *
Wash-Buffer A		
Wash-Buffer B		
Direct Block Solution	Store for up to 6 months at - 20°C. *	Store for up to 5 days at 4°C. * Could be refrozen.
SDB ₁₀₀₀		Do not use SDB that has been thawed for more than 4 hours.
CDB-b		Do not use CDB-b that has been thawed for more than 4 hours.
CDB		Do not use CDB that has been thawed for more than 4 hours.
PCR-Masternix	Store for up to 6 months at - 80°C. *	Thaw immediately prior to usage. Do not refreeze or reuse. Protect against light. Do not use after more than 30 min.
Imperacer® conjugate		Thaw immediately prior to usage. Do not refreeze or reuse. Do not use after more than 20 min.

* Provided this is within the expiration date of the kit

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash-Buffer A - Dilute 50 ml of Wash-Buffer A concentrate into 950 ml distilled water to prepare 1000 ml of Wash-Buffer A.

Wash-Buffer B - Dilute 50 ml of Wash-Buffer B concentrate into 950 ml distilled water to prepare 1000 ml of Wash-Buffer B.

Imperacer® conjugate - Dilute Imperacer® conjugate concentrate according to protocol into CDB-B, SDB or serum to prepare a ready-to-use solution.

Instruments, Materials:

- Digital pipette 1-10, 10-100 and 100-1000 μ l
- Multichannel pipette, 50-300 μ l
- Microplate compatible PCR-thermocycler. For reproducing the described results use Imperacer® enabled real-time PCR cycler
- Orbital shaker for microplates
- Heating device / oven, if NOT using recommended microplates
- Siliconised 1.5 ml cups
Its recommended to use siliconised cups in ultrasensitive applications for minimization of unspecific binding of low antigen or reagent concentrations against the surface of the cups
- Filtertips applicable for used pipettes
To avoid cross-contamination use filtertips for each pipetting and washing step during the assay
- Precision Wipes
- Cling Film

PRECAUTIONS

The kit is manufactured with the relevant safety and quality regulations.

Wear laboratory clothes and protective gloves during the sample preparation and during the whole kit procedure to avoid an infection from the samples. (The kit itself contains no infectious material!). Contaminated areas must be cleaned with disinfection solution.

Samples and kit components must be disposed in accordance with the common laboratory safety regulation.

ASSAY PROCEDURE

Bring wash buffers to room temperature before use. Chimera Direct Block, SDB₁₀₀₀ should be stored on ice during assay procedure. It is recommended to assay all samples, standards and controls in duplicate.

1. Imperacer®-Assay

Previous to PCR-amplification, the antigen to be detected is immobilized on a microplate surface and coupled with the DNA marker.

1.1. Immobilization of capture antibody and antigen

It is recommended to immobilize the antigen through a capture antibody. Additionally biotinylated protein could be immobilized (1.1.2.) and detected directly with the Imperacer® conjugate as control.

1.1.1. Immobilization of capture antibody and coupling with the antigen

Coating of the capture antibody:

1. Dilute capture antibody in coating buffer. Use concentrations according to manufacturers instructions or typical for ELISA assays (e.g. 20 µg/ml) as well as 10-fold or 100-fold dilutions of this start concentration for assay optimization.
2. Add 30 µl/well of the capture antibody dilution into the microplate modules. Seal the modules with cling film and incubate for 12 - 48h at 4°C.
3. Wash three times for one min with 240 µl/well Wash-Buffer A at room temperature and orbital shaking.
4. Add 240 µl/well of the direct block solution and incubate for at least 1 minute at room temperature and orbital shaking.

Note: All washing steps can be carried out either manually, using multichannel pipettes, half-automatically (e.g. with ImmunoWash8, Nunc) or fully automated. The following protocol gives instructions for manual washing. For a model protocol of automated washing, see below, "TECHNICAL HINTS" or contact chimera biotec for technical advice.

Binding of the antigen through the capture antibody:

1. If the antigen to be analyzed is contained in a biological matrix (e.g. a serum), the sample could be diluted 1:1 in sample dilution buffer (SDB). For pure antigen solutions, no dilution is necessary. During assay optimization, the dilution step could be varied between 1:1 and 1:10 for the suppression of matrix effects.
2. Wash the capture-antibody coated and blocked modules twice for 30 sec and twice for 4 min under orbital shaking with 240 µl/well Wash-Buffer B.
3. Add 30 µl of the diluted samples to each well. Incubate the samples for 25 min at room temperature and orbital shaking. All samples should be carried out in double determination.
4. If the antigen to be analyzed is biotinylated, carry on with step 1.3 otherwise with step 1.2.

1.1.2. Direct immobilization of the biotinylated protein as a control

Additionally biotinylated protein (antigen or primary antibody) can be immobilized directly on the plate and detected with the Imperacer® conjugate (incubation as described below in 1.3.) as positive control.

1. Make a dilution row of the biotinylated protein (e.g. biotin-BSA) in coating buffer. Reasonable concentrations are between 0.01 - 10,000 amol/well.
2. Add 30 µl/well of the dilution row into the microplate modules. Seal the modules with cling film and incubate for 12 - 48h at 4°C. Subsequently, wash the protein coated modules three times for one min each with 240 µl/well Wash-Buffer A at room temperature and orbital shaking.
3. Incubate the modules with 240 µl/well direct block solution for at least 1 minute at room temperature and orbital shaking.
4. After blocking the modules carry on with step 1.3.

1.2. Application of a primary antibody

If you use an indirect assay (page 4, picture b) a biotinylated primary antibody has to be immobilized to your antigen. Otherwise, proceed directly with step 1.3.

1. After incubation of the antigen (1.1.1.) wash twice for 30 sec and twice for 4 min under orbital shaking with 240 µl/well Wash-Buffer B at room temperature.
2. Incubate with 30 µl/well of the biotinylated primary antibody in CDB. Use concentrations according to manufacturers instructions or typical for ELISA assays (e.g. 10 ng/ml) as well as 10-fold or 100-fold dilutions of this start concentration for assay optimization. Incubate for 25-45 min at room temperature / orbital shaking.

The efficiency of this kind of indirect assays could be enhanced by the direct linking of the primary antibody with the DNA marker. These kind of custom Imperacer® conjugates are provided by Chimera Biotec.

1.3. Imperacer® Conjugate Immobilization:

1. After incubation of the antigen (1.1.) and, if necessary, the biotinylated primary antibody (1.2.), wash twice for 30 sec and twice for 4 min with 240 µl/well Wash-Buffer B at room temperature and orbital shaking. Dilute 3 µl Imperacer® conjugate in 597 µl CDB-b (biotin-free) immediately before use (1:200). For adaptation of the assay to different matrixes and sample collection techniques, the dilution could be varied between 1:30 and 1:1000.
2. Incubate with 30 µl/well of Imperacer® conjugate for 25-45 min at room temperature / orbital shaking. (Keep the residual dilution conjugate for the PCR control.)
3. Wash seven times with Wash-Buffer B (4 x 30 sec, 3 x 4 min) and twice for 1 min with Wash-Buffer A.

2. Real-time PCR

Thaw the frozen PCR-Mastermix immediately prior to usage and keep it on ice.

1. Pipette 30 µl of the PCR-Mastermix into each well. It is suggested to include a PCR negative control into a blank well (see below “Controls”).
2. Seal the modules with the Adhesive Foil. Apply pressure to the foil from the top by using a precision wipe or an Adhesive Seal Applicator tool (Chimera, cat.-no. 24-007). Remove the modules from the frame by applying gentle pressure from below to the modules. Make sure that no PCR-solution is spilled against the cover.

If a centrifuge with a rotor compatible to 96 well microplates is available, it is recommended to spin down the PCR-mix after sealing.

An alternative way for step 2 is to transfer the empty modules previous to the application of the PCR-Mastermix from the frames to a 96 well rack (thus avoiding to spill any contains).

3. Clean the bottom of the modules with precision wipes to remove any dust from handling.
4. Transfer the sealed modules into the real-time PCR cycler.
5. Place an "Optical Cover Compression Pad" or a similar spacer-mat (according to manufacturers instructions) on the sealed modules. Make sure that the openings in the pad will overlay with the positions of the wells.
6. Close the lid and start the PCR. Make sure that the heated lid is operating and set the correct well volume in the PCR program.

Carry out a PCR according to the program in **table 1**. Setting-up FAM (emission at 518 nm) as fluorophor. Make sure that measurement is carried out at 50°C in the PCR-cycle. Deactivate the measurement of ROX as standard if necessary.

<i>Time</i>	<i>Temperature</i>	<i>Repeats</i>
5 min	95°C	1x
30 sec	72°C	
12 sec	95°C	40x
30 sec	50°C	

Table 1: PCR-program for real-time Imperacer®.
Deactivate the measurement of ROX as standard if necessary.

PCR Controls:

It is recommended to include two empty wells in a clean, non-coated microplate module with 30 μ l PCR-Mastermix as a negative control and another two wells with 30 μ l of the PCR mastermix spiked with 1 μ l of the residual diluted Imperacer® conjugate as a PCR-positive control.

CALCULATIONS OF RESULTS

The real-time PCR-cycler records the increase of the normalized fluorescence signal (dR) for each cycle during DNA amplification. Subsequent to the run, an automatic baseline correction is applied by the software of the instrument. In the next step, the software automatically calculates the threshold cycle (Ct), which represents the first PCR cycle at which the reporter signal exceeds the signal of a given uniform "Threshold", manually set it in the phase where signal increases linearly (typically 100 -1000). Use a half-logarithmic plot of log dR against cycle number to choose the correct threshold value. To render an easy comparison of data obtained from real-time Imperacer® and conventional ELISA, the problem has to be circumvented that Ct values are inversely proportional to antigen concentrations (NC has the highest numerical value) while ELISA signals are directly proportional to antigen concentrations (NC has the smallest numerical value). Therefore, it is recommended to calculate ΔCt values by subtracting the Ct values obtained for each signal from the total number of cycles carried out in the experiment. This purely mathematical conversion facilitates the comparison of the data.

For the data analysis use an appropriate program (e.g. MS Excel). For each sample and/or standard analysed in duplicate calculate mean values and standard deviation of ΔCt . For quantification, plot ΔCt of the calibration curve standards against the log and carry out a linear regression. The resulting equation will be used for the determination of the unknown samples concentration.

TECHNICAL HINTS

If an automatic microplate washer is available (will be provided in Imperacer® Instrumentation), the washing procedure in the Imperacer® protocol could also be carried out automatically. The following washing programs should be used as a template for automatic washing (adjust programs according to the properties of the available washer):

Standard washing step (Buffer B):

Bottomwash	100 µl
Wash	2x 250 µl
<i>After each step 10 s waiting period</i>	
Wash	2x 1000 µl (rinse wash)
Aspirate	

PCR washing step:

<i>1. Buffer: B</i>	
Bottomwash	100 µl
Wash	4x 250 µl
<i>After each step 10 sec. waiting period</i>	
Wash	3x 1000 µl (rinse wash)
<i>2. Buffer: A</i>	
Bottomwash	100µl
Wash	2x 1000 µl (rinse wash)
Aspirate	

For working with toxic reagents

For Imperacer®, the usage of the half-automatic washing apparatus is possible. With these, Imperacer® could be performed in a closed system inside a sterile bench. Use separate washers for Wash-Buffer A and Wash-Buffer B.

If the microplate modules are incompatible with the heating block of the intended real-time PCR cycler or standard microplates are used.

Perform the Imperacer®-Assay as described. Subsequently, proceed for real-time PCR as following:

1. Seal the plate including 35 µl of the added PCR-mastermix with Adhesive Foil.
2. Heat the plate for 5 minutes at 95°C by placing them inside a heating device / oven.
3. Transfer the hot supernatant (including the denaturated DNA marker) from the plate to a set of vessels compatible with your real-time PCR cycler. If possible use a multichannel pipette. Work fast and try to avoid cross-contamination by spilling of the hot PCR-mix.
4. Seal/close your PCR-vessels and proceed regarding to manufactures instructions using the PCR program described in **table 1**.

This procedure allows the combination of Imperacer® and almost any cycler. The additional pipetting step, however, leads to a variation in assay performance.

High background / General assay problems

In case of high non-specific background signals, all reagents and especially the PCR Mastermix should be exchanged with new aliquots. In case of no specific signals, control experiments for the PCR and the proteins should be carried out.

Additionally check the following possible sources of cross-contamination / errors:

- Did you use an Optical Cover Compression Pad?
- Are you using filtertips?
- Are you using gloves for protecting your samples?
- Are you using fresh aliquots for every experiment?
- Are you elongating suggested assay times?
- Are you using appropriate vessels for each dilution step (e.g. sterile vessels, siliconised cups, vessels with correct volume)?
- Are you mixing each dilution thoroughly (e.g. by using a vortex)?
- Are you touching the modules and/or the adhesive foil previous to amplification without subsequent cleaning?
- Are you performing positive/negative controls and double determinations for the control of errors?

- Did the reagents remain non-frozen for long times previous to application?
- Check your washing procedure:

If you are washing manually, please check that you are not spilling reagents during emptying of the microplate wells into other wells. It is highly recommended not only to pour the wells out, but additionally to forcefully push them against a tissue which is changed at each washing/emptying step.

If you have additional questions regarding the assay protocol and/or the general Imperacer® procedure, please contact:

info@chimera-biotec.com

for advice.

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